Genetic appraisal of the *Culex sitiens* subgroup in Australasia: major arbovirus vectors

by

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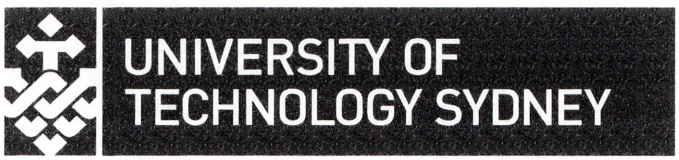

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CERTIFICATE OF AUTHORSHIP/ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Stéphane Hemmerter

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PREFACE

Malaria, Japanese encephalitis, Chikungunya, Dengue fever… are responsible for millions of deaths. These diseases have one common point: they are transmitted by mosquitoes. These mosquitoes continually spread to new environments and at the same time become insecticide resistant. This creates a real threat of global disease transmission and presents a real challenge for science. What could be more exciting than undertaking a PhD on the other side of the world investigating the major virus vectors of Australasia?

English is not my first language so you will understand that I was a little confused when I arrived in Australia and I heard that people drink Cab Sav, go to Woop Woop and even adult men have barbies. And who can explain why when your alarm goes off, it goes on? Or why sun baking is not a new environmentally friendly way of cooking a cake?

When I arrived four years ago from France I never imagined I would write a thesis, produce several papers, participate in exciting workshops, organize a symposium in Barcelona and even win a prize for "outstanding presentation" at the Mosquito Control Association of Australia / Arbovirus Research Australia conference in English! I came to do a challenging PhD, knowing nothing about mosquito vectors nor evolutionary biology and only speaking broken English so it was a great honour to finish my PhD and win the Dr. Elizabeth N. Marks Award.

The question is would Dr Marks be concerned because a molecular phylogenetics won the prize?

Dr. Elizabeth Nesta Marks (Pat) was one of Australia's leading entomologists and malaria experts, she spent much of her life studying and classifying mosquitoes and discovering new species. Today I think she would have totally embraced molecular techniques and phylogenetics as supplementary tools to reveal mosquito diversity. I would have loved to have met her (with a chocolat chaud and croissants of course) and I am sure we

would both agree that phylogenetics is exciting and useful. Morphological identification will always be an important way to discriminate mosquitoes. There is no competition between the two fields but there is a synergy. Phylogenetics should not be seen as obscure and it is no more difficult to learn than anything else.

My PhD provides a good example of how collaboration can be fruitful: collaboration between disciplines (virology, entomology and evolutionary biology), collaboration between places (1 territory, 4 states and 4 countries) and collaboration between institutions (universities, public health institutes and the Department of Defense).

Finally I would like to thank everyone involved for their support, advice, guidance and for helping me to discover and love my research and Australia in general.

Stéphane

PREFACE

Le paludisme, l'encéphalite japonaise, Chikungunya, dengue, ... sont responsables de millions de morts. Ces maladies ont un point commun: elles sont transmises par les moustiques. Ces moustiques colonisent continuellement de nouveaux environnements tout en devenant résistants aux insecticides. Cela crée une menace réelle de transmission de maladie à l'échelle mondiale et représente un réel défi pour la science. Qu'est-ce qui pourrait être plus excitant que de faire un doctorat à l'autre bout du monde pour étudier les principaux vecteurs de virus de l'Australasie.

L'anglais n'est pas ma langue maternelle, alors vous comprendrez ma surprise lorsque je suis arrivé en Australie et j'ai entendu dire que les gens boivent du « Cab Sav », vont à « Woop Woop » et que même les hommes adultes ont des « Barbies». Et qui peut m'expliquer pourquoi, lorsque votre réveil s'allume, on dit qu'il s'éteint? Pourquoi « cuire au soleil » n'est pas une nouvelle façon écologique de faire cuire un gâteau?

Quand je suis arrivé de France, il y a quatre ans, je n'aurais jamais pu imaginer que j'allais écrire une thèse, produire plusieurs articles scientifiques, participer à des formations passionnantes, organiser un symposium à Barcelone et même gagner le prix pour une "excellente présentation" lors de la conférence de l' «association de contrôle des moustiques d'Australie et de la recherche sur les arbovirus » et cela en anglais! Je suis venu pour faire un doctorat ambitieux, ne sachant rien sur les moustiques, ni en biologie de l'évolution et en parlant un mauvais anglais. C'est donc pour moi un grand honneur de finir ma thèse en remportant le Prix Dr. Elizabeth N. Marks.

La question est de savoir si le Dr. Marks s'inquièterait du fait qu'un phylogénéticien moléculaire ait remporté le prix qui porte son nom?

Le Dr. Elizabeth Nesta Marques (Pat) a été reconnue comme l'une des plus grandes entomologistes et spécialiste du paludisme en Australie. Elle a passé une grande partie de sa

vie à étudier, à classer les moustiques et à découvrir de nouvelles espèces. Aujourd'hui, je pense qu'elle aurait totalement adopté les nouvelles techniques moléculaires et phylogénétiques en tant qu'outil permettant de révéler la diversité des moustiques. J'aurais bien aimé l'avoir rencontré (devant un chocolat chaud et avec des croissants bien sûr) et je suis sûr que nous aurions été tous les deux d'accord sur le fait que la phylogénétique est un domaine passionnant. La caractérisation morphologique sera toujours un moyen important de discrimination des moustiques. Il n'y a pas de concurrence entre les deux domaines mais il y a une synergie. La Phylogénétique ne doit pas être considérée comme obscure et elle n'est pas plus difficile à apprendre que tout autre chose si elle est bien expliquée

Ma thèse est un bon exemple de la façon dont une collaboration peut être fructueuse: la collaboration entre les disciplines (virologie, entomologie et biologie évolutive), la collaboration entre les lieux (un territoire, quatre états et quatre pays) et la collaboration entre les institutions (universités, instituts de santé publique et la Département de la défense).

Enfin, je tiens à remercier tout le monde pour leur soutien, conseils et assistance. Merci de m'avoir fait découvrir et aimer mes recherches ainsi que l'Australie.

Stéphane

TABLE OF CONTENTS

JOURNAL PUBLICATION

Hemmerter, S., Slapeta, J. & Beebe, N. 2009, Resolving genetic diversity in Australasian *Culex* mosquitoes: incongruence between the mitochondrial cytochrome c oxidase 1 and nuclear acetylcholine esterase 2, *Molecular Phylogenetics and Evolution* **50**:317-325.

Hemmerter, S., J. Slapeta, A. van den Hurk, R. Cooper, P. Whelan, R. Russell, C. Johansen, and N. Beebe. 2007. A curious coincidence: mosquito biodiversity and the limits of the Japanese encephalitis virus in Australasia. BMC Evolutionary Biology **7**:100.

Johansen, C. A., V. Susai, R. A. Hall, J. S. Mackenzie, D. C. Clark, F. J. May, S. Hemmerter, D. W. Smith, and A. K. Broom. 2007. Genetic and phenotypic differences between isolates of Murray Valley encephalitis virus in Western Australia, 1972-2003. Virus Genes **35**:147-154.

SYMPOSIUM ORGANISATON AND CHAIRING

Symposium: "What would/should we do with a million DNA barcodes?" with Prof. Dr. Axel Meyer; Annual meeting of the Society for Molecular Biology and Evolution, (Barcelona, Spain, 5-8 June 2008)

INVITED PRESENTATIONS

Faculty of Veterinary Science seminar
"How mosquito phylogenetics helps us to understand the distribution of Japanese encephalitis"
University of Sydney (Sydney, Australia, 4th December 2008)

Workshop Evolution across Scales (integrating Genomics, Geosciences and Evolutionary Biology).
"How phylogenetics resolves epidemiological mystery?"
(University of Potsdam, Germany, 26th -27th May 2008)

Invited speaker at the Leibniz Institute for Zoo and Wildlife Research, Prof. Dr. Simone Summer laboratory seminar (Berlin, Germany, 28th May 2008)

CONFERENCE PRESENTATIONS

Stéphane Hemmerter, Jan Šlapeta, Nigel W. Beebe
25th Annual Royal North Shore Hospital·UTS·USYD·Kolling Scientific Research Meeting
How biotechnology resolves an epidemiological mystery? (Sydney, Australia, 18th-19th November 2008)

Stéphane Hemmerter, Jan Šlapeta, Nigel W. Beebe
7th Conference of the Mosquito Control Association of Australia / 10th Arbovirus research in Australia Symposium
How mosquito phylogenetics helps us to understand the distribution of Japanese encephalitis (Coffs Harbour, Australia, 15th-19th September 2008)

Stéphane Hemmerter, Jan Šlapeta, Nigel W. Beebe
A curious coincidence: mosquito biodiversity and the limits of the Japanese encephalitis virus in Australasia.
11th Congress of The European Society for Evolutionary Biology (Uppsala, Sweden, 20-25 August 2007)

Stéphane Hemmerter, Jan Šlapeta, Nigel W. Beebe
Insight into the limits of the Japanese encephalitis virus through a better understanding of the biodiversity of its mosquito vector.
54th Annual Conference of the Genetics Society of AustralAsia (Sydney, Australia, 26-29 June 2007)

Stéphane Hemmerter, Jan Šlapeta , Andrew F. van den Hurk, Robert D. Cooper, Peter I. Whelan, Richard C. Russell, Cheryl A. Johansen, Nigel W. Beebe
Phylogeography of Culex annulirostris and Culex palpalis: arbovirus vector of Australia and Papua New Guinea.
7th Conference of the Mosquito Control Association of Australia (Cairns, Australia, 15-18 August 2006)

POSTER PRESENTATION

Hemmerter, S., Slapeta, J., Beebe, N.
A curious coincidence: mosquito biodiversity and the limits of the Japanese encephalitis virus in Australasia.
Annual meeting of the Society for Molecular Biology and Evolution (Barcelona, Spain, 5-8 June 2008)

AWARDS

John W. Hambley Prize for the best UTS postgraduate student at the 25th Annual Royal North Shore Hospital·UTS·USYD·Kolling Scientific Research Meeting (Sydney, Australia, 18th-19th November 2008)

Dr. Elizabeth N. Marks Award for "outstanding student presentation" at the Mosquito Control Association of Australia (Coffs Harbour, Australia, 15th-19th September 2008)

Student Plenary Prize at the 54th Conference of the Genetics Society of Australasia (Sydney, Australia, 26-29 June 2007)

LIST OF FIGURES

LIST OF TABLES

Chapter 3: Genetic diversity of *Culex* mosquitoes with reference to the southern limits of the Japanese encephalitis virus

Chapter 4: Acetylcholine esterase 2 as a nuclear marker for the *Culex sitiens* subgroup

Chapter 5: Development of a Molecular Identification tool to discriminate members of Culex annulirostris complex

LIST OF ABBREVIATIONS

Abbreviation	Full name
ace	acetylcholinesterase gene
AChE	acetylcholinesterase protein
ae	*Aedes*
AMI	Australian Army Malaria Institute
ANIC	Australian National Insect Collection
ASPCR	allele-specific PCR
BFV	Barmah Forest virus
BLAST	basic local alignment search tool
BOLD	Barcode of Life Data systems
bp	base pair
CDC	Centre for Disease Control
COI	cytochrome oxidase I
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Cx	*Culex*
DMSO	dimethyl-sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
EDTA	ethylene diamine tetra acetic acid
EtOH	ethanol
EVS	encephalitis virus surveillance
ITS	internal transcribed spacer
JEV	Japanese encephalitis virus
Kac	Potassium acetate
kb	kilobases
KUNV	Kunjin virus
min	minutes
MRN	Mosquitoes Reference Number
mtDNA	mitochondrial DNA
ml	millilitre
ML	Maximum Likelihood
MVEV	Murray Valley encephalitis virus
mya	million years ago
NCBI	National Centre for Biotechnology Information
NNDSS	National Notifiable Diseases Surveillance System
NSW	New South Wales
nrDNA	nuclear DNA
NT	Northern Territory
NUMTS	nuclear mitochondrial DNA
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RAPD	Randomly Amplified Polymorphic DNA
rDNA	ribosomal DNA
RFLP	Restriction Fragment Length Polymorphisms

PAMSA	PCR amplification of multiple specific allele
PNG	Papua New Guinea
rpm	revolutions per minute
RRV	Ross River virus
SA	South Australia
SDS	sodium dodecyl sulfate
Tm	melting temperature
UV	ultraviolet
W	Watt
WA	Western Australia
WNV	West Nile virus
www	world wide web

ABSTRACT

Culex annulirostris Skuse, *Culex palpalis* Taylor and *Culex sitiens* Wiedemann are members of the *Culex sitiens* subgroup that exist in Australasia. They are widely distributed and regularly comprise over the half of mosquitoes collected from arbovirus surveys and field collections in Australia and PNG. From these mosquitoes, *Cx. annulirostris* is the major vector of endemic arboviruses in Australia and is also responsible for the establishment of the Japanese encephalitis virus (JEV) in southern Papua New Guinea (PNG) as well as JEV incursions into northern Australia.

Papua New Guinea and mainland Australia are separated by a small stretch of water, the Torres Strait, and its islands. While there has been regular JEV activity on these islands, JEV has not established on mainland Australia despite an abundance of its vector *Cx. annulirostris* and porcine amplifying hosts. Despite the public health significance of this mosquito and the fact that its adults show overlapping morphology with close relative *Cx. palpalis*, its evolution and genetic structure remain undetermined. I address a hypothesis that there is significant genetic diversity in *Cx. annulirostris* and that the identification of this diversity will shed light on the paradox that JEV can cycle on an island 70 km from mainland Australia while not establishing in Australia itself.

My study assessed the biodiversity within *Cx. annulirostris* and *Cx. palpalis* using a extensive collection of mosquitoes and analysing both mitochondrial (*COI*) and a nuclear (*ace-2*) markers using phylogenetic reconstruction and hypothesis testing. I provide evidence of subdivision of these two species into several geographically delimited lineages, which indicates the presence of highly divergent populations or cryptic species. However I found lack of congruence between nuclear and mitochondrial markers. While I found eight divergent lineages geographically restricted within *Cx. annulirostris* and *Cx. palpalis* at the mitochondrial level, only four lineages appeared at the nuclear level. Two lineages appeared consistent using both markers: *Cx. palpalis* S-AUS and *Cx.annulirostris* PNG1.

Notably, the southern limit of the *Cx. annulirostris* PNG1 lineage coincides exactly with the current southern limit of JEV activity in Australasia suggesting that biological variation in this lineage may be the key to why JEV has not yet established yet on mainland Australia.

I assessed the discrimination power of *COI* barcoding for *Culex* mosquitoes and conclude that DNA barcoding using *COI* may actually overestimate the diversity of *Culex* mosquitoes in Australasia and should be applied cautiously with support from nuclear DNA, such as the polymorphic ace-*2* gene.

Finally I designed and developed a PCR-based diagnostic tool to discriminate *Cx. annulirostris* PNG1 from its morphologically identical relative. This tool will greatly assist identification and surveillance of *Cx. annulirostris* PNG1 into Australia mainland in the future as it may move south into Australia as a consequence of climate change or population expansion.

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